REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
	FINAL REPORT	15 APR 07 - 30 NOV 09

4. TITLE AND SUBTITLE
NOVEL BIOCATALYSTS COMBINING THE SPECIAL ASSEMBLY PROPERTIES OF S-LAYER PROTEINS AND THE FUNCTIONALITY OF ENZYMES OF EXTREMOPHILES

5a. CONTRACT NUMBER

5b. GRANT NUMBER
FA9550-07-1-0313

5c. PROGRAM ELEMENT NUMBER
61102F

6. AUTHOR(S)
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5d. PROJECT NUMBER
2312

5e. TASK NUMBER
EX

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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DEPTOF NANOBIOTECHNOLOGY
MUTHGASSE LL, A-1190 VIENNA, AUSTRIA

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
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875 NORTH RANDOLPH ST, SUITE 325, ROOM 3112
ARLINGTON, VA 22203-1768

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
A = APPROVED FOR PUBLIC RELEASE; DISTRIBUTION IS UNLIMITED

AFRL-OSR-VA-TR-2012- 0033

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Methods for organizing functional materials at the nanometer level are essential for the development of novel fabrication technique. One of the most relevant areas of research in nanobiotechnology concerns technological utilization of self-assembly systems, wherein molecules spontaneously associate into reproducible supramolecular structures. Being composed of a single protein or glycoprotein species, bacterial cell surface layers (S-layers) represent the simplest biological membrane developed during evolution.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(Include area code)*
				44	

FINAL PERFORMANCE REPORT

Title: Novel Biocatalysts Combining the Special Assembly Properties of S-Layer Proteins and the Functionality of Enzymes of Extremophiles (BIOCAT)

Agreement Award No.: FA9550-07-1-0313
Reporting period: April 15th, 2008 to April 14th, 2010

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1. ABSTRACT

Methods for organizing functional materials at the nanometer level are essential for the development of novel fabrication technique. One of the most relevant areas of research in nanobiotechnology concerns technological utilization of self-assembly systems, wherein molecules spontaneously associate into reproducible supramolecular structures. Being composed of a single protein or glycoprotein species, bacterial cell surface layers (S-layers) represent the simplest biological membrane developed during evolution. Based on the remarkable intrinsic feature of S-layer proteins to self-assemble and the possibility for genetic modifications, S-layer proteins were exploited as component for the development of novel immobilized biocatalysts based on fusion proteins comprising S-layer proteins of Bacillaceae and enzymes from extremophiles. The produced nanoscale biocatalysts combined the intrinsic feature of S-layer proteins to self-assemble into a spatial predictable way with the remarkable stability and robustness of monomeric or multimeric extremozymes. By exploiting the self-assembly property of the S-layer protein moiety, the chimeric protein was used for spatial control over display of enzyme activity on planar and porous supports. The results obtained clearly demonstrate that S-layer based bottom up self-assembly systems for functionalizing solid supports with a catalytic function could have significant advantages over processes based on random immobilization of sole enzymes.

2. SPECIFIC TASKS OF THE PROJECT

1. Design and expression of fusion proteins based on S-layer proteins and enzymes of extremophilic organisms in *Escherichia coli*
2. Isolation, purification and refolding of the different fusion proteins
3. Investigation of the enzymatic activity of the different fusion proteins
4. Recrystallization of the S-layer fusion proteins in suspension or on different artificial solid supports
5. Development of novel immobilized biocatalysts based on biologically active S-layer/extremozyme fusion proteins
6. Improvement of the stability of the biocatalysts by chemical cross-linking of the S-layer portion of the fusion proteins
7. Comparison of enzyme stability of S-layer/extremozyme fusion proteins with those of extremozymes covalently linked to S-layer protein lattices
8. Exploitation and dissemination of the results

3. STATUS OF EFFORT

	Objectives	Status
1.	Design and expression of fusion proteins based on S-layer proteins and enzymes of extremophilic organisms in *Escherichia coli*	✓ (completed)
2.	Isolation, purification and refolding of the different fusion proteins	✓ (completed)
3.	Investigation of the enzymatic activity of the different fusion proteins	✓ (completed)
4.	Recrystallization of the fusion proteins in suspension or on different artificial solid supports and investigation of the accessibility of the fused enzyme portion by TEM and AFM	✓ (completed)
5.	Development of novel immobilized biocatalysts based on biological active S-layer/extremozyme fusion proteins	✓ (completed)
6.	Improvement of the stability of the biocatalysts by chemical cross-linking of the S-layer portion of the fusion proteins	✓ (completed)
7.	Production of biocatalysts based on extremozymes covalently linked to S-layer protein lattices	✓ (completed)
8.	Exploitation and dissemination of the results	work in preparation

4. NANOBIOTECHNOLOGY WITH S-LAYERS

Crystalline bacterial cell surface layers, termed S-layers (*1*), are one (*2*) of the most commonly observed outermost cell envelope structures of Bacteria and Archaea. These monomolecular isoporous structures, composed of a single protein or glycoprotein species represent the simples protein membranes developed during evolution (*2*). Most important, isolated S-layer (glyco)proteins possess the intrinsic property for recrystallization into isoporous lattices in suspension and at a broad range of surfaces (e.g., polymers, silicon, metals) and interfaces (e.g., air-liquid interface, lipid films, liposomes).

Since one of the key challenges in biotechnology and material sciences is the technological utilization of self-assembly systems wherein molecules spontaneously associate into defined supramolecular structures, S-layers have a great potential as patterning elements. The well-defined arrangement of functional groups on S-layer lattices and the repetitive physicochemical properties down to the nanometer scale allows the binding of functional molecules (e.g., enzymes, antibodies, antigens, ligands) and nanoparticles with unsurpassed spatial control. Moreover, S-layers can be used as structural basis for a biomolecular

construction kit involving all major species of biological molecules (proteins, lipids, glycans, nucleic acids and combinations of them).

The possibility of modifying and changing the natural properties of S-layer proteins by genetic engineering techniques and to incorporate specific functional domains while maintaining the self-assembly capability has led to new types of enzyme membranes, affinity structures, ultrafiltration membranes, microcarriers, biosensors, diagnostic devices, biocompatible surfaces, vaccines as well as targeting, delivery and encapsulation systems.

Presently, the most important line of development concerns genetic manipulation of S-layer proteins and glycoproteins. For nanobiotechnological utilization of self-assembly systems, S-layer technology was advanced by the construction of genetically engineered S-layer fusion proteins that comprised (i) the N-terminal cell wall anchoring domain, (ii) the self-assembly domain, and (iii) a functional sequence (*3-5*). Numerous studies have clearly demonstrated that S-layer proteins incorporating specific functional domains of proteins (e.g., enzymes, antibodies, antigens, ligands, mimotopes) maintain the capability to assemble into coherent lattices on a great variety of solid supports. This strategy for "nanocontrolled" funcionalization of surfaces leads to new enzyme and ultrafiltration membranes, affinity structures, ion-selective binding matrices, microcarriers, biosensors, diagnostics, biocompatible surfaces, mucosal vaccines, and encapsulation systems (*6, 7*). Due to this construction principle, the functional sequences are aligned at a predefined distance in the nanometer range on the outermost surface of the S-layer lattice and thus, remain available for further binding reactions (e.g., substrate binding, antibody binding).

The S-layer protein SbpA of mesophilic *Lysinibacillus sphaericus* CCM 2177 consists of 1268 amino acids, including a 30 amino acids long signal peptide (*8*). By producing various C-terminally truncated forms and performing surface accessibility screens, it became apparent that amino acid position 1068 is located on the outer surface of the square lattice and that this C-terminally truncated form fully retained the ability to self-assemble into a square S-layer lattice with a center-to-center spacing of the tetrameric morphological units of 13.1 nm (*8*). Therefore, the C-terminally truncated form $rSbpA_{31\text{-}1068}$ was used as base form for the construction of various S-layer fusion proteins. An advantage of the SbpA system for nanobiotechnological applications is, that the recrystallization is dependent on the presence of calcium ions, thus allowing control over lattice formation (*9*).

5. BIOCAT ACCOMPLISHMENTS

5.1. The S-layer-based enzyme immobilization system

Currently used immobilization methods are based on the adsorption and covalent binding of enzymes to water-insoluble carriers, the incorporation of enzymes into semi-permeable gels, and the enclosing of enzymes in polymer membranes (Fig. 1a–c). Regarding enzyme immobilization, the advantages offered by the S-layer self-assembly system are (i) the requirement of only a simple, one-step incubation process for site-directed immobilization without preceding surface activation of the support, (ii) the provision of a cushion to the enzyme through the S-layer moiety of the fusion protein preventing denaturation, and, consequently, loss of enzyme activity upon immobilization, (iii) the principal applicability of the "S-layer tag" to any enzyme, and (iv) the high flexibility for variation of enzymatic groups within a single S-layer array by co-crystallization of different enzyme/S-layer fusion proteins (Fig. 1d).

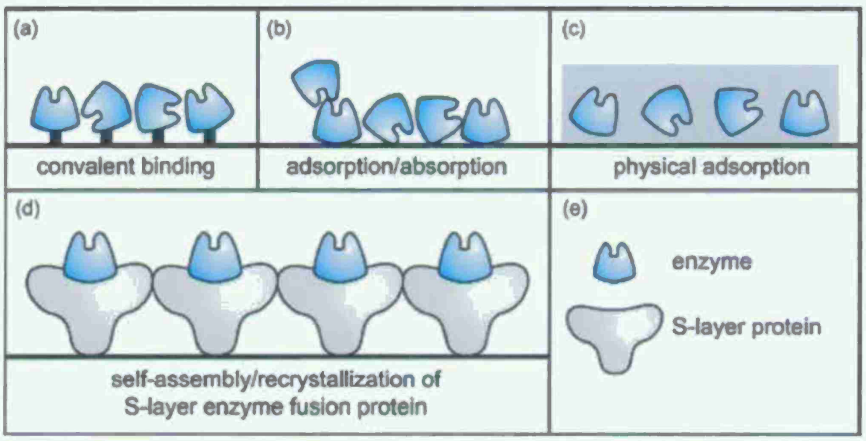


Fig.1.Comparison of different enzyme immobilization methods. (a) Random immobilization via covalent binding; (b) random adsorptive binding; (c) random physical adsorption within a 3D gel structure; (d) novel approach for site directed immobilization of an enzyme via the S-layer self-assembly technique, allowing orientated and dense surface display of the enzyme in its native conformation and ensuring accessibility for the substrate; (e) legend.

5.2. S-layer-based biocatalysts carrying monomeric extremozymes

a) rSbpA/LamA fusion protein

Overexpression, isolation and purification

In a first approach, a fusion protein based on the S-layer protein SbpA of *Lysinibacillus sphaericus* CCM 2177 and the enzyme laminarinase (LamA) from *Pyrococcus furiosus* was cloned and overexpressed in *E. coli* BL21 (DE3)star. The extremophilic enzyme LamA is an

endoglucanase displaying its main hydrolytic activity on the β-1,3-glucose polymer laminarin. It is extremely thermostable (half life time at 100 C, 16 h) and thermoactive (temperature optimum, 100–105 C), and, as a particular property, it is remarkably resistant to denaturation retaining a significant extent of its secondary structure in 8 M guanidinium hydrochloride (GHCl). These properties are accompanied by a notable stability at extremely low pH (~3). Due to the robustness of the chosen enzyme, the rSbpA/LamA construct is well suited for the evaluation of the S-layer self-assembly system for enzyme immobilization.

The designed S-layer fusion protein carries a C-terminal VSV-G tag, interspacing Ser-Ala-Ser-Ser-Gly-Gly-Gly-Gly-Ser-Ala, was fused via a Gly-Gly linker (Fig. 2).

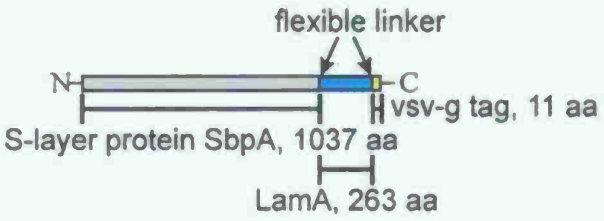


Fig. 2. Cartoon of rSbpA/LamA

In *E. coli* cells induced for expression, the fusion protein accumulated as inclusion bodies within the cell cytoplasm. An additional band of a 2-h expression culture appearing on an SDS-PAGE gel upon Coomassie staining corresponded to the calculated molecular mass for rSbpA/LamA of 141.9 kDa (Fig. 2). For isolation of rSbpA/LamA, the biomass harvested from a 4-l fermentation was treated with B-PER® reagent. rSbpA/LamA was extracted by treatment with 5 M GHCl (3 ml g^{-1} inclusion bodies) and finally enriched by GPC to high purity with an overall yield of 600 mg l^{-1}. The integrity of the S-layer fusion protein was confirmed by binding of anti-VSV-G peroxidase conjugate to the C-terminal VSV-G tag in a Western blot experiment (Fig. 3).

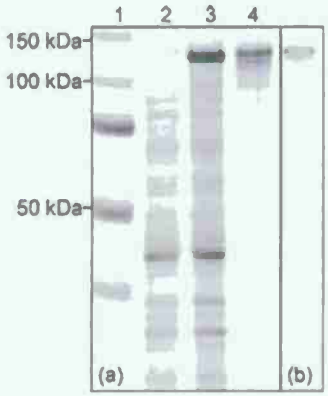


Fig. 3 Overexpression and purification of the rSbpA/LamA fusion protein. (a) SDS-PAGE analysis after Coomassie staining. Lane 1, Benchmark ladder; lane 2, *E. coli* BL21star(DE3) expression culture harboring plasmid pET28a/*sbpA/lamA* before induction; lane 3, after 2 h of induction; lane 4, purified rSbpA/LamA fusion protein. (b) Western blot analysis of rSbpA/LamA using anti-VSV-G-peroxidase conjugate.

Self-assembly and recrystallization properties of rSbpA/LamA As shown by transmission electron microscopy of negatively stained preparations, rSbpA/LamA had the capability to self-assemble in suspension into monomolecular, regular arrays (Fig. 4a). Self-assembled and recrystallized rSbpA/LamA exhibited the square lattice structure characteristic of native SbpA with a center-to-center spacing of tetrameric morphological units of 13.1 nm. The defined orientation of rSbpA/LamA was demonstrated via labeling with anti-VSV-G-FITC conjugate after recrystallization on cellulose microspheres. Green luminescence of microspheres indicated exposure of the enzymatic moiety at the surface (Fig. 4b), while uncoated particles and glass slides showed no fluorescence (not shown).

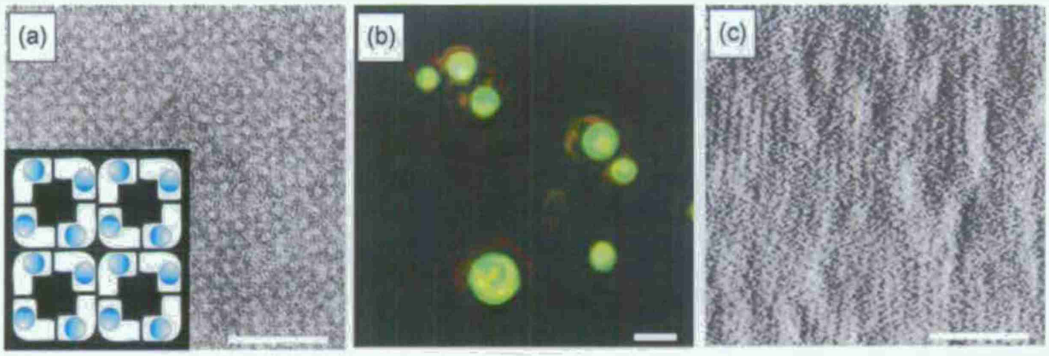


Fig. 4 (a) TEM image of a negatively stained preparation of rSbpA/LamA self-assembled in solution into a monomolecular array (bar, 100 nm). Inset showing the square lattice symmetry of the S-layer enzyme fusion protein with a lattice constant of 13.1 nm (enzyme moiety in blue); (b) fluorescent microscopic image of microspheres coated with rSbpA/LamA after binding of an anti-VSV-G FITC conjugate (bar, 3 μm), indicating surface exposure of the enzymatic group; (c) AFM deflection image of the S-layer enzyme fusion protein after recrystallization on a silicon wafer, measured in contact mode in aqueous solution (bar, 100 nm).

Determination of the enzyme activity of rSbpA/LamA

The enzyme activity of the S-layer fusion protein was determined by its ability to release glucose from the polysaccharide laminarin and compared to the sole enzyme. Water soluble rSbpA/LamA protein as present in the absence of calcium ions revealed a specific activity of 344 U mg^{-1}, while the value for sole LamA was 727 U mg^{-1}. Thus, the S-layer fusion protein had approximately 50% of the specific enzyme activity of the sole enzyme. The difference in activity might be due to different diffusion rates between the substrate and sole LamA and the fused enzyme, respectively, or to partial denaturation of the SbpA moiety at the high temperature (*i.e.*, 90°C) applied during the enzyme assay.

Exploitation of the SbpA lattice for enzyme immobilization on planar supports

The performance of the S-layer fusion protein upon immobilization on diverse supports, including silicon wafers and glass slides was investigated (Table 1). Lowry assays of rSbpA/LamA protein lattices that have been detached from the silicon wafers revealed that 0.004 nmol of rSbpA/LamA occupied a surface area of 1 cm^2, which corresponded to the calculated amount of a tetrameric protein monolayer (four molecules rSbpA/LamA per 171.61 nm^2). Monolayer formation was also confirmed by AFM evidence (Fig. 4c). The specific enzyme activity determined for the rSbpA/LamA lattices recrystallized as a monolayer on a silicon wafer was 774 U mg^{-1}, which matched the specific enzyme activity of sole LamA. Obviously, alignment of the catalytic epitopes within the fusion protein lattice resulted in better accessibility for the substrate than when provided as soluble rSbpA/LamA fusion protein. Generally, immobilization transfers enzymes in a randomly orientated, insoluble state with a limited reaction space and may lead to partial denaturation at the sites of adhesion to the support. It is conceivable that with S-layer fusion proteins, the S-layer moiety acts as a cushion preventing denaturation of the enzyme moiety upon immobilization.

Table 1. Enzyme activity (mM/cm^2) of S-layer fusion protein rSbpA/LamA and sole enzyme LamA when immobilized on different solid supports

Planar solid supports	LamA[a] 33,123[c]	rSbpA/LamA[b] 141,919[c]
Silicon wafer	0.1±0.0	6.1±0.4
Epxoy-activated glass slides	3.4±0.1	6.2±0.4
Glas slides	0.4±0.1	6.8±0.1
Membranes		
PES membrane	0.1±0.0	5.1±0.2
Cellulose acetate membrane	2.7±0.4	4.8±0.2
Sartobind®Epoxy 75 membrane	13.1±0.3	16.5±0.5
Cellulose membrane	1.1±0.6	11.6±1.3
Polypropylene membrane	0.0±0.0	3.2±0.5

[a]Sole enzyme LamA was covalently bound to epoxy-activated glass slides and to the Sartobind®Epoxy 75 membrane. On all other solid supports, it was attached adhesively.
[b]In the absence of Ca^{2+} ions, monomeric rSbpA/LamA was covalently bound in random orientation to epoxy-activated glass slides. On all other solid supports, crystallized arrays were formed due to the presence of calcium ions.
[c]Molecular weight.
[d]Measurements were performed in quadruplicate and standard deviations were calculated.

Furthermore, LamA and rSbpA/LamA were immobilized in a random fashion on epoxy-activated glass slides and the enzyme activity of the composites was determined. For direct comparison with the results obtained for sole LamA, which *per se* is randomly immobilized, calcium was omitted for random immobilization of rSbpA/LamA. As expected, rSbpA/LamA revealed higher enzyme activity than sole LamA, with values being approximately twice as high (for accurate data including standard deviations, see Tab. 1).

The additional advantage of SbpA as a fusion partner for LamA through provision of a regular display matrix for the enzymatic function was demonstrated after formation of the rSbpA/LamA lattice on non-activated glass slides. These composites revealed approximately twice the glucose release of LamA immobilized on an epoxy-activated glass surface. When using non-activated glass slides for adhesive immobilization of LamA, the S-layer fusion protein was even 17-fold more active than sole LamA (Tab. 1). This finding clearly demonstrates that supports with low binding capacity for an enzyme can be utilized when applying the S-layer fusion protein approach.

Immobilization of rSbpA/LamA on membranes

Enzyme immobilization on membranes constitutes an interesting area of applied research, because such microporous composites favor easy flow of substrates and products, and may be integrated in more complex processes, where combination of a catalytic function with a conventional filtration function is required. Among the attractive features of enzyme-immobilized membrane reactors are easy control, straightforward scaling up, prolonged enzyme activity, high flow rates and reduced costs. For comparative immobilization studies, rSbpA/LamA and sole LamA were adhesively bound to several commercially available membranes and the glucose release per membrane unit area was determined. For all membranes tested, there was a significantly higher enzyme activity when the rSbpA/LamA protein was immobilized as opposed to sole LamA immobilization. Best results were obtained for the polyethylensulfonate (PES) membrane with an approximately 50-fold higher enzyme activity (Tab. 1).

Effects of temperature and chemicals on the catalytic activity of immobilized rSbpA/LamA in comparison to sole LamA

S-layer lattices can be stabilized with the homobifunctional cross-linker Dimethyl pimelinimidate (DMP). Comparative studies on the chemical resistance and temperature resistance of cross-linked rSbpA/LamA and sole LamA was investigated upon immobilization

on glass slides. It was crucial to note that cross-linking of the S-layer fusion protein led only to a 10% reduction of enzyme activity compared to the native (non-cross-linked) rSbpA/LamA monolayer. When immobilized on glass slides in a cross-linked state, rSbpA/LamA maintained a much higher enzyme activity upon exposure to 6 M urea and 0.1 M NaOH at 25 °C and to a temperature of 90 °C. Cross-linked rSbpA/LamA withstood even treatment with 6 M urea for 8 h at 25 °C without loss of enzyme activity (Fig. 5a) and protein content per square unit. In contrast, LamA covalently bound to epoxy-activated glass slides showed a rapid reduction of glucose release with retaining only 15% of its original value under the same conditions.

Investigation of the effect of 0.1 M NaOH, which is commonly used as sanitizing agent, on the enzyme activity revealed that on glass slides neither native rSbpA/LamA nor covalently bound LamA was stable at 25 °C. The loss of enzymatic activity of rSbpA/LamA corresponded to desorption of the S-layer moiety from the surface. However, cross-linking with DMP could retain half of the LamA activity of the S-layer fusion protein after approximately 30 min of exposure (Fig. 5b).

The rSbpA/LamA protein was also superior to sole LamA when the enzyme activity was determined upon exposure to 90 °C. The sole enzyme LamA exhibited a half life time of 3.8 h, whereas the S-layer fusion protein retained around half of the enzymatic activity after 40 h (Fig. 5c). The protective effect of the S-layer moiety prevented the loss of enzymatic activity, however it could not withstand the temperature exposure itself resulting in gradual denaturation and desorption from the surface. A protein leakage of 60% was determined after exposure to 90 °C for 40 h.

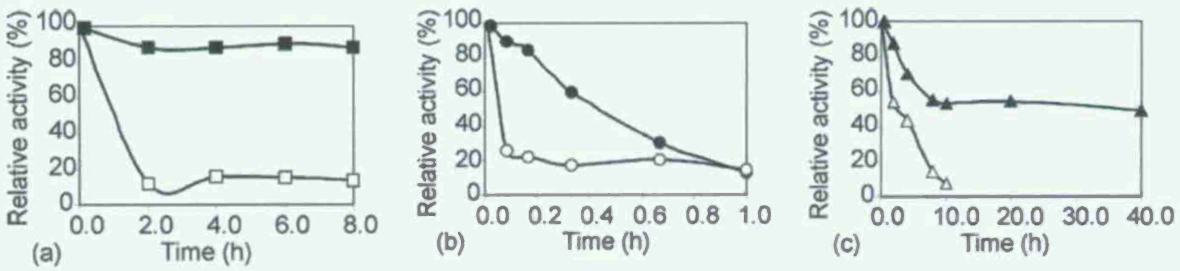


Fig. 5. Comparative stability studies on rSbpA/LamA recrystallized and cross-linked on glass slides and LamA covalently bound onto epoxy-activated glass slides. Enzyme activity upon exposure to (a) 6 M urea (■ rSbpA/LamA, □ LamA), (b) 0.1 M NaOH (● rSbpA/LamA, ○ LamA), and (c) 90°C (▲ rSbpA/LamA, △LamA). Enzyme activity is given as percentage of original activity of the respective composite.

In 20% ethanol, the "stability" of either enzyme system (sole LamA and S-layer fusion protein) was constant for 14 days, without decrease of enzyme activity. Freeze dried samples of rSbpA/LamA immobilized on glass slides fully maintained the enzyme activity upon rewetting, whereas sole LamA covalently bound glass slides revealed a 26% loss of activity upon regeneration. Autoclaving of both, rSbpA/LamA and LamA coated glass slides completely abolished the enzyme activity.

Investigation of the operational stability and protein leakage of rSbpA/LamA

The ability of the immobilized enzyme to retain activity after repeated exposure to the analyte is an important technological aspect of enzymes. The time course of enzyme activity of recrystallized and cross-linked rSbpA/LamA protein compared to adhesively bound LamA after repeated use is given in Fig. 6. The S-layer fusion protein retained 31% of its original enzyme activity after eight cycles within 20 days, whereas the activity of sole LamA dropped to 10% of its original value, when assayed under identical conditions. With the leakage of the S-layer fusion protein during recycling studies being only 4%, the S-layer system fulfils the requirement for separation of the enzyme system from reaction mixtures.

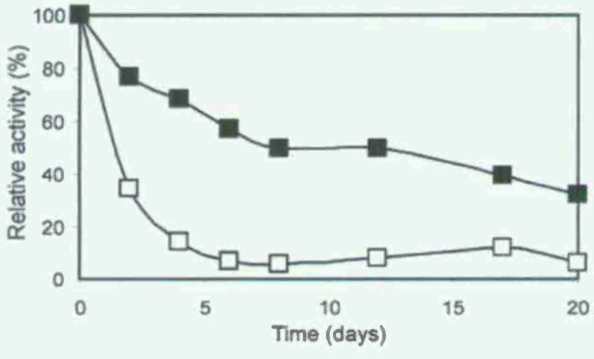


Fig. 6. Recycling of LamA immobilized as S-layer enzyme fusion protein (■) on silicon wafers in comparison to adhesively bound sole enzyme LamA (□). Enzyme activity is given as percentage of original activity of the respective composite.

To account for the storage stability, which is another important issue for the application potential of an enzyme, rSbpA/LamA recrystallized on glass slides was stored in MilliQ® water at 25°C with addition of a preservative and the enzyme activity was monitored over a period of 3 weeks. During that time, no decrease of enzyme activity of either rSbpA/LamA or sole LamA was detectable.

b) rSbpA/Lac fusion protein

Laccases belong to a large family of multicopper oxidases capable of oxidizing a wide range of inorganic and aromatic compounds, while reducing molecular oxygen to water (*10*). Classical laccases are considered to be exclusively associated with plants and fungi. Due to the much higher thermostability of bacterial laccases, biocatalysts based on these enzymes may have advantageous properties compared to classical laccases (*11*). The laccase Lbh1 of the alkaliphilic *Bacillus halodurans* C-125 shows laccase-like activity, oxidizing 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS), 2,6-dimethoxyphenol (DMP) and Syringaldazine (SGZ). The enzyme shows an alkaline pH optimum with SGZ as the substrate and is stimulated rather than inhibited by chloride. Since Lbh1 is optimally active at alkaline pH, it may be expected that this enzyme is also less susceptible to inhibition by other anions (*12*). The electrocatalytic properties of the laccase Lbh1 open up the potential of using this enzyme for the development of cathodes for enzyme-catalyzed bio fuel cells. Therefore, the fusion protein rSbpA/Lac, comprising the truncated S-layer protein SbpA of *Lysinibacillus sphaericus* CCM 2177, a glycine linker and the C-terminally fused laccase Lbh1, was constructed (Fig. 7). The aim was to obtain a completely covered electrode with recrystallized rSpbA/Lac and consequently, an oriented exposed enzyme array on the electrode surface maximal accessible for the substrate. As alternative strategies, we considered a) an rSbpA/Lac fusion protein with a cysteine residue introduced at the very N-terminal end of the protein sequence, as well as b) chemical linkage of the sole laccase to the S-layer lattice.

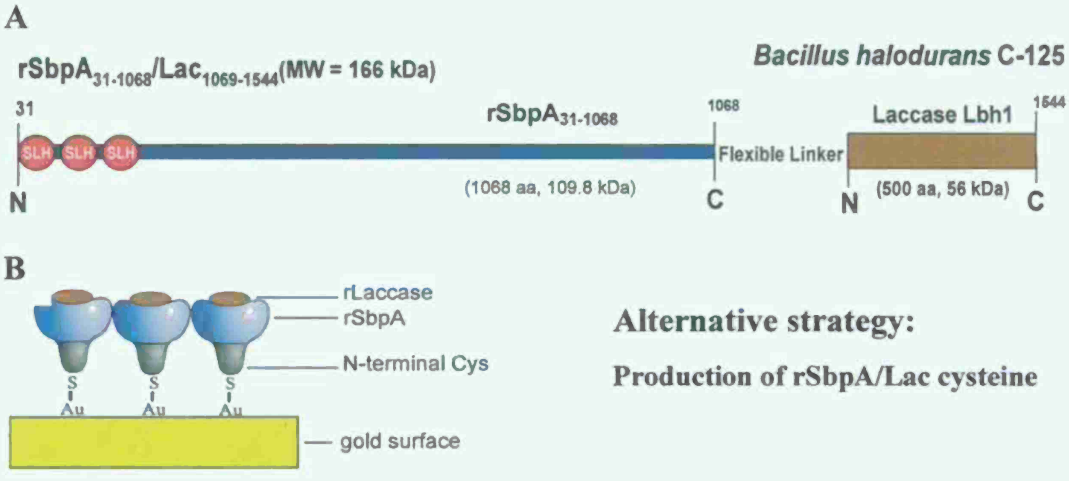


Fig. 7. Schematic representation of the fusion protein comprising the truncated S-layer protein SbpA ($rSbpA_{31-1068}$) and the laccase (Lac) combined by a six aa long glycine linker (A). Alternatively, the fusion protein $_{Cys}$rSbpA/Lac provides a strong interaction with the gold surface of the electrode mediated by the introduction of a cysteine residue (B).

Cloning, expression, isolation and purification of the SbpA/Lac fusion protein and the sole enzyme

Within the Biocat project, the S-layer/extremozyme fusion proteins carrying S-layer protein SbpA and the laccase of *B. halodurans* C-125 (rSbpA/Lac) as well as the sole enzyme were cloned and heterologously expressed in *E. coli* (Fig. 8). For the construction of expression vectors, first intermediate vectors were made by using the primer pairs *SbpA*37/*BamHI-SbpA*$_{1068}$*rev* and *NcoI-*$_{cys}$*SbpA-for*/*BamHI-SbpA*$_{1068}$*-rev* (Tab. 2) to generate a fragment of the *spbA* gene encoding amino acids 31-1068 of the SbpA sequence and amino acids 31-1068 with an introduced N-terminal cysteine residue ($_{Cys}$31-1068) of the SbpA sequence, and cloning it into the *Nco*I and *BamH*I sites of pET-28a(+). The template for adding the sequence encoding the six amino acids long linker (5'-GGA TCC GGT GGG GGA GGG-3') via the primer pair *BamH*I-*Linker*6*Lac-for/Not*I-*lac-rev* (Tab. 2) was obtained by amplifying the *laccase* nucleotide sequence with the primer pair *Lac-for/Not*I-*lac-rev* (Tab. 2). During PCR, the restriction sites *BamH*I and *Not*I were introduced at the 5' and 3' ends, respectively.

For expression, all recombinant plasmids were established in *E. coli* BL21 Star™ (DE3) (Invitrogen). After induction of gene expression by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG), samples of cultures from *E. coli* BL21 Star™ (DE3) were taken at distinct points of time and analyzed by SDS-PAGE (Fig. 8 and 9). The proteins could be isolated from the insoluble cytoplasmic fraction and purified by gel-permeation chromatography (GPC) (Fig. 9, 10 and 11).

Table 2. Oligonucleotide primers used for amplification in PCR; underlined: overhang, **bold**: restriction sites, *italicized*: start and stop codon

Primer	[1.1] *Primer Sequence 5'to 3'*
Lac-forw	*ATG*AAAAAATCATATGGAGTGAT
BamHI-Linker$_6$*Lac-forw*	CGC**GGATCC**GGTGGGGGAGGG*ATG*AAAAAATCATATGGAGTGAT
NotI-Lac-rev	GGCC**GCGGCCGC***TTA*CTCAGGCATATTTGGAAT
*SbpA*37	Ilk *et. al.* 2002
NcoI-$_{Cys}$*SbpA-for*	CGGAATT**CC*ATG***GCGCAAGTAAACTGCGACTATAAC
BamHI-SbpA$_{1068}$*-rev*	CGC**GGATCC**TTCTGAATATGCAGTAGTTGCTGC

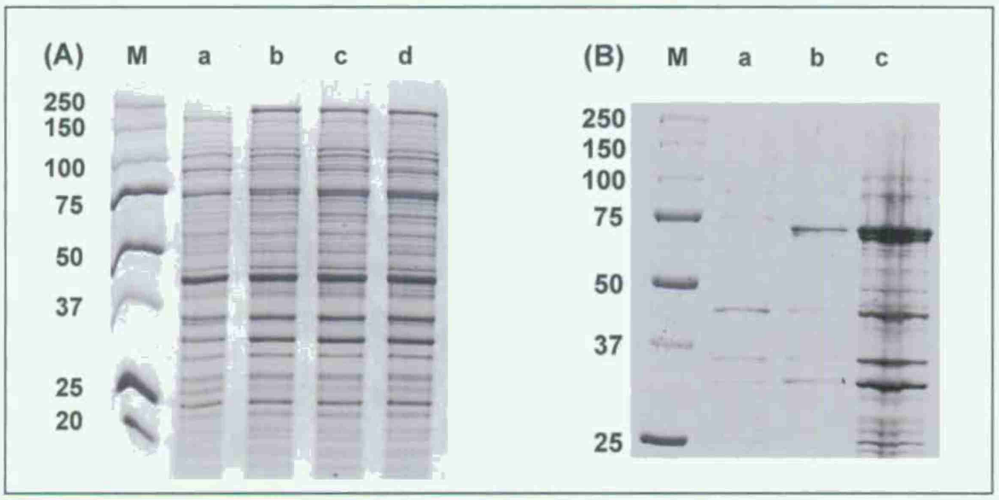


Fig. 8. SDS-PAGE analysis and Coomassie staining of SDS extracts from *E. coli* BL21 Star™ (DE3) expression cultures containing (A) the pET28a/*sbpA/lac* gene construct before (lane a), and 1, 2 and 3 h after induction of *sbpA/lac* expression (lanes b, c and d).
(B) SDS-PAGE patterns of whole-cell extracts of *E. coli* BL21 Star™ (DE3) containing the pET28a/*lac* gene construct before (lane a), and 2 and 3 h after induction of *lac* expression (lanes b and c). Selected molecular masses are given in kDa.

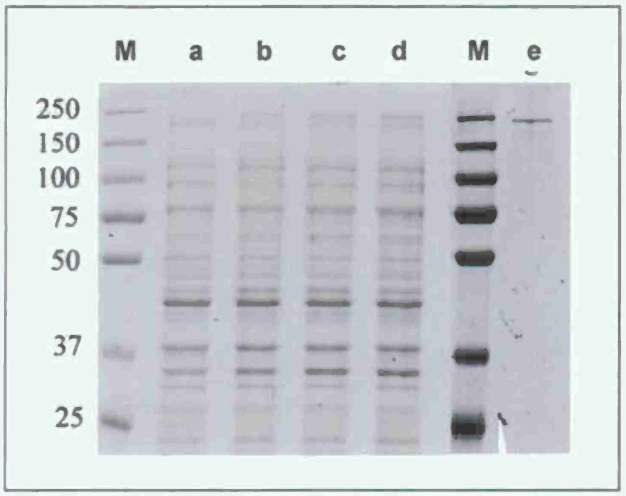


Fig. 9. SDS-PAGE analysis and Coomassie staining of SDS extracts from *E. coli* BL21 Star™ (DE3) expression cultures containing the pET28a/$_{cys}$*sbpA/lac* gene construct before (lane a), and 1, 2 and 3 h after induction of *sbpA/*$_{cys}$*lac* expression (lanes b, c and d). Lane e: isolated $_{cys}$SbpA/Lac fusion protein.

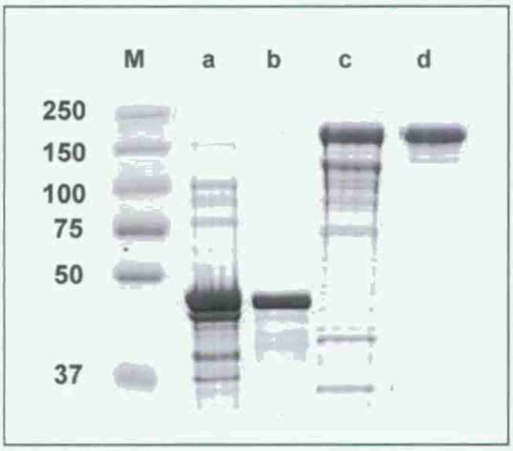


Fig. 10. SDS-PAGE analysis and Coomassie staining of SDS extracts from the laccase and the rSbpA/Lac fusion protein after isolation (lanes a and c) and GPC (lanes b and d). Selected molecular masses are given in kDa.

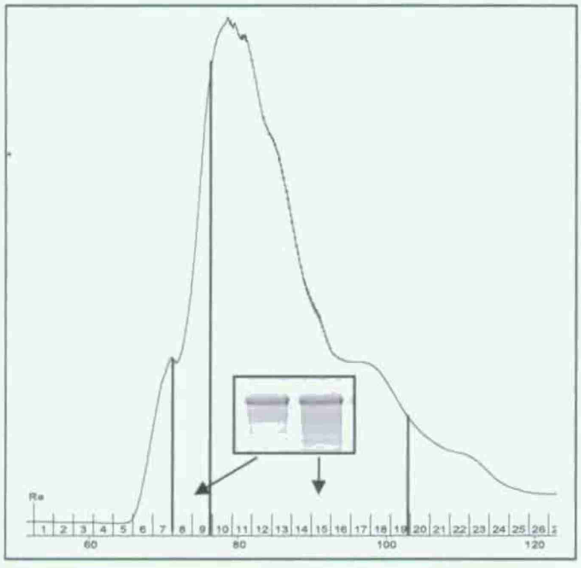


Fig. 11. Purification of rSbpA/Lac by gel permeation chromatography. The protein bands demonstrate the purified proteins in the respective pooled fractions

Determination of the enzymatic activity of the laccase and rSbpA/Lac

Specific enzyme activity of the laccase and the fusion proteins was determined for different substrates: 2,6-Dimethoxyphenol (DMP, ε_{468}=14,800 l mol^{-1} cm^{-1}), Syringaldazine (SGZ, ε_{530}=64,000 l mol^{-1} cm^{-1}), 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS, ε_{420}=38,000 l mol^{-1} cm^{-1}) and Hydrochinone (HQ). Control measurements without the respective protein were performed to correct a possible chemical oxidation of the substrates. Quantification of enzymatic activity was done under different conditions (pH, temperature and buffer). The pH of the buffers used in the assays was adjusted to 30°C, 40°C and 45°C, respectively. Spectrophotometrically, as well as electrochemically, the highest specific laccase activity of the S-layer fusion protein in solution was determined for pH 7.5 to 7.6 at 45°C, indicating that fusion of the extremozyme to the S-layer did not influence the enzymatic properties of the laccase (Tab. 3). The specific activity of rLac as well as rSbpA/Lac increases with temperature. Remarkably, the rSbpA/Lac fusion protein showed 10 x higher solubility at RT than sole rLac.

Preparation of the proteins for the activity assays

The lyophilized proteins (rLac and rSbpA/Lac) were dissolved in 7 M guanidine hydrochloride (GHCl) in 50 mM Tris/HCl buffer (pH 7.2) and dialysed against Aqua purificata (A. purif.; Milli-Q grade; resistance, > 18.2 MΩ cm^{-1}) containing 1 mM copper sulphate. In order to get rid of the copper in the protein solution, an additional dialysis step against A. purif. was done. Precipitated protein was removed by centrifugation and the protein concentration in the clear supernatant was determined by UV absorbance at 280 nm using the sequence-derived extinction coefficient.

Spectrophotometric measurements: rSbpA/Lac and rLac in solution

The reaction mixture (100 mM MOPS buffer, supplemented with 100 mM NaCl and the respective protein solution) was pre-incubated at 30°C or 45°C. The reaction was started by adding the substrate to a final concentration of 1 mM (DMP and ABTS) or 22 µM (SGZ) (*12*). After incubation for 5 minutes, the concentration of the oxidized substrates was calculated using a photometer adjusted to the respective wavelength. 50 mM Citrat buffer was used in the reaction mixture for the determination of the activity with ABTS as the substrate.

First, a screening in order to determine the best conditions for the activity tests were performed (Fig. 12a). Subsequently, the main studies were carried out with DMP, because it turned out to be the most efficient substrate for this laccase and the fusion protein (Tab. 3A) (*12*).

Stability tests: proteins in solution

Investigation of storage stability revealed that soluble rLac as well as rSpbA/Lac stored in A. purify. at 4°C retained 70% of its original enzyme activity after two weeks and 20% of its original activity after eight weeks.

Electrochemical measurements: rSbpA/Lac and rLac in solution

Hydroquinone is a preferred substrate of the laccase and its digestion is electrochemically quantifiable by linear sweep voltammetry. Laccase oxidizes hydroquinone to quinone by releasing two protons and two electrons. In the presence of oxygen, the enzyme forms water. First of all the temperature (RT and 40°C) as well as the pH (pH 6 to 7.8) were varied (Fig. 12b). This screening work was carried out on so-called screen-printed electrodes (Drop Sens). The working electrode was gold, the reference electrode silver and the counter electrode platinum. All experiments were performed in potassium phosphate buffer (20 mM) with 100 mM sodium chloride at various pH. The applied stepwise (10 mV/s) potential of the voltammograms started from 0 and ended at 0.4 V. The occurring current peak (0.2 to 0.25 V) was evaluated in the absence and in the presence of laccase in solution (*13*). The competition between the laccase and the working electrode is visible owing to the decrease of the anodic current peak after addition of the enzyme. If no laccase is added, the current peak remains unchanged. With respect to Faradays` law, the current is proportional to the oxidized molar amount of the substrate. However, only the activity (units) and not the specific activity (units per mg of enzyme) could be determined by this technique due to the unaware exact mass of the enzyme at the electrode interface. The best activity for the fusion protein as well as for the sole enzyme was at 40°C at pH 7.6 (Tab. 3B).

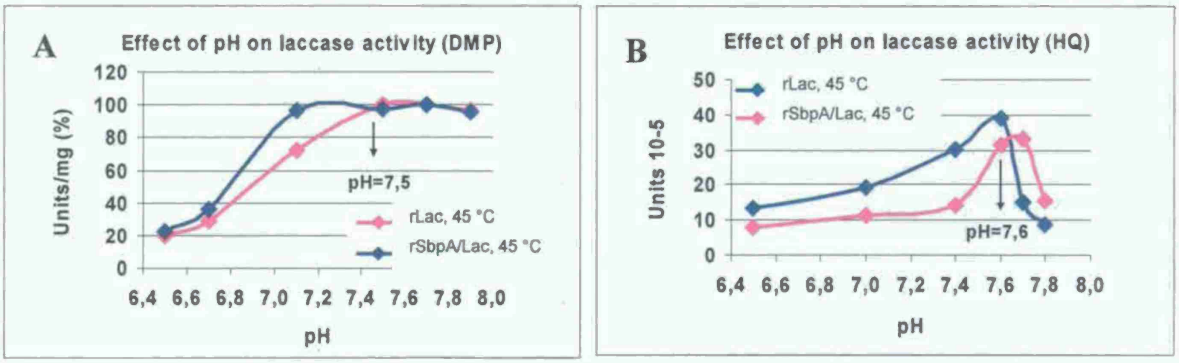


Fig. 12. pH-dependent activity profile of recombinantly produced sole rLac and of the S-layer fusion protein rSbpA/Lac for two different substrates (DMP and HQ) determined photometrically (A) and electrochemically (B)

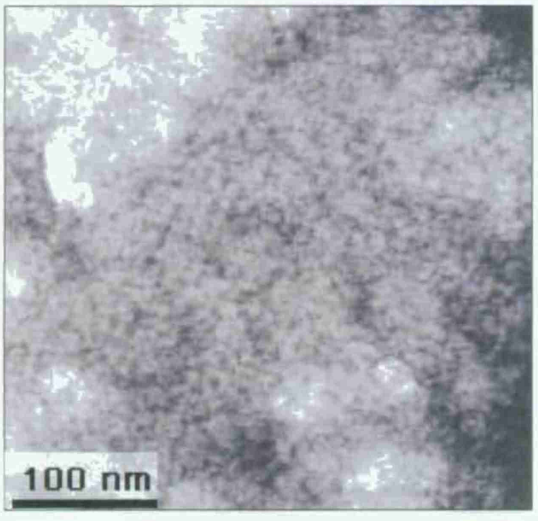
Table 3. Maximum laccase activity obtained with different substrates in solution

A Substrate	T (°C)	pH	**rLac** (units*/mg)	**rSbpA/Lac** (units*/mg)
2,6-dimethoxyphenol (DMP)	30	7.5	10.21 +/- 0.052	9 +/- 0.33
DMP	45	7.5	**30.91**+/- 0.78	**25.42** +/- 0.076
Syringaldazine (SGZ)	45	7.9	0.175 +/- 0.007	0.4 +/- 0.1
ABTS	45	3.0	n. d.	0.69
B Substrate	T (°C)	pH	**rLac** (units*10^{-5})	**rSbpA/Lac** (units*10^{-5})
Hydrochinone (HQ)	RT	7.7	0.24 +/- 0.04	1.04 +/- 1.12
HQ	40	7.6	**3.14** +/- 2.47	**3.90** +/- 1.42

A. Analysis by spectrophotometry. B. Electrochemical method (linear sweep voltammetry).
* 1 U corresponds to the amount of laccase that oxidizes 1 µmol substrate per minute under standard assay conditions. Taking different molecular weights into account; value represents U/mg calculated to the amount of enzymatic groups.

Investigation of the self-assembly and recrystallization properties of rSbpA/Lac for coating of electrodes

Investigation by transmission electron microscopy of negatively stained preparations revealed that rSbpA/Lac had the capability to self-assemble in suspension into monomolecular arrays (Fig. 13) and to recrystallize on solid supports (Fig. 14). In case of rSbpA/Lac, coating of surfaces showed more patch-like structures and did not result in closed monolayers exhibiting the same lattice symmetry on the solid support. We considered the introduction of a cysteine residue at the very N-terminal end of the fusion protein. The motivation was to obtain a strong interaction between the S-layer moiety and the gold surface and consequently a forced orientation of the enzyme which is a prerequisite for immobilized enzymes on cathodes. Results showed that the enzymatic activity of $_{Cys}$rSbpA/Lac was weak and also the adsorption of this cysteine-mutant to gold surfaces compared to the rSbpA/Lac (checked by atomic force micrsoscopy, AFM; not shown) was not satisfying. However, when rSbpA/Lac was recrystallized on a gold surface for determining the defect area and compared the results with recrystallized SbpA, a complete coverage of the electrode surface was observed. Therefore, we proceeded our studies with the rSbpA/Lac fusion protein.



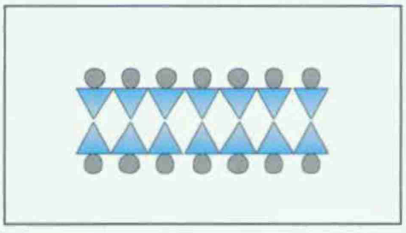
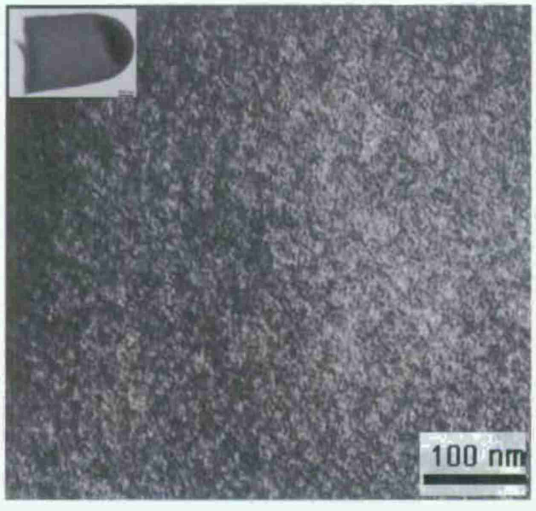


Fig. 13. TEM image of a negatively stained preparation of a double layered rSbpA/Lac self-assembly product formed in solution



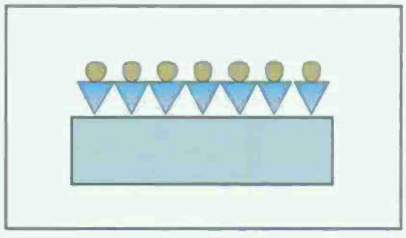


Fig. 14. TEM image of a negatively stained preparation of rSbpA/Lac fusion proteins recrystallized as a crystalline monolayer on peptidoglycan containing sacculi of *Ly. sphaericus* CCM 2177

Determination of defect area of rSbpA/Lac covered QCM-D gold sensor:

The adsorption of rSbpA/Lac on gold as substrate was compared with the wild-type SbpA, which exhibits excellent recrystallisation properties. Information on the blocking effect or defect area of recrystallized S-layer proteins can be obtained by determining the charge transfer resistance R_{ct} compared to a bare gold electrode in the presence of an electro-active species. For the measurement of R_{ct}, electrochemical impedance spectroscopy (EIS) was utilized. The ratio of the detected R_{ct} of gold and S-layer protein is an indication of the amount of area-wide defects within the spread layer (Fig. 15). Recrystallized S-layer proteins reduce the accessibility of the electrode area for electroactive species. Hence, R_{ct} is increasing (*14*).

$$\Delta R_{ct} = \frac{R_{ct(Au)}}{R_{ct(gold+S-layer)}} \times 100$$

Fig. 15. Equation 1

EIS measurements were carried out on an electrochemical quartz-crystal microbalance cell (E-QCM-D) consisting of three electrodes: the working electrode was the QCM-D gold sensor itself; a platinum plate auxiliary electrode and a KCl saturated Ag/AgCl reference electrode. As supporting electrolyte, a 10 mM phosphate buffer containing 100 mM NaCl (PBS) of pH 7.4 was used. The redox system $K_3Fe(CN)_6/K_4Fe(CN)_6$ was present in solution at 10^{-3} M concentration in all experiments. Impedance measurements were carried out at a DC bias voltage of 0.5 V, were an electron transfer process occurs. The frequency range started from 100 000 Hz to 10 MHz and a small AC sinusoidal voltage of 15 mV between the working electrode and the counter electrode was applied. The obtained impedance spectra

were fitted to the classical equivalent circuit of Randles. The determined change in the charge transfer of SbpA was 0.36 % and that of SbpA/Lac 0.51 %. These results indicate that even though no square lattice structure is visible (checked by AFM), the rSbpA/Lac covers the entirely electrode surface.

Spectrophotometric measurements: proteins recrystallized on a gold crystal (QCM-D)

QCM-D measurements were carried out using a Q-sense instrument. By spreading the protein solutions (proteins solubilized in crystallization buffer: 0.5 mM Tris-HCl buffer, 10 mM $CaCl_2$, pH 9.0) in situ on a QCM-D crystal (crystallization time: 180 min), a decrease of the frequency adverse to an increase of the dissipation upon protein adsorption on the gold sensor was shown (Fig. 16). Two sensors were coated with SbpA; the first one was used as reference surface and the second sensor was used for the immobilization of rLac (amine coupling by EDC/NHS to the SbpA-layer). The third sensor was functionalized with rSbpA/Lac. The masses of the adsorbed layers were calculated by the Sauerbrey equation $m=(c*f)/n$(c:17.7 ng Hz^{-1} cm^{-2}) (*15*). Afterwards, the temperature was set to 45°C. Followed by collecting the oxidized substrate in tubes, the proteins immobilized on the QCM-D crystals were incubated with the reaction mixture (100 mM MOPS buffer, supplemented with 100 mM NaCl and 1 mM DMP). The amount of converted DMP was determined spectrophotometrically (Tab. 4).

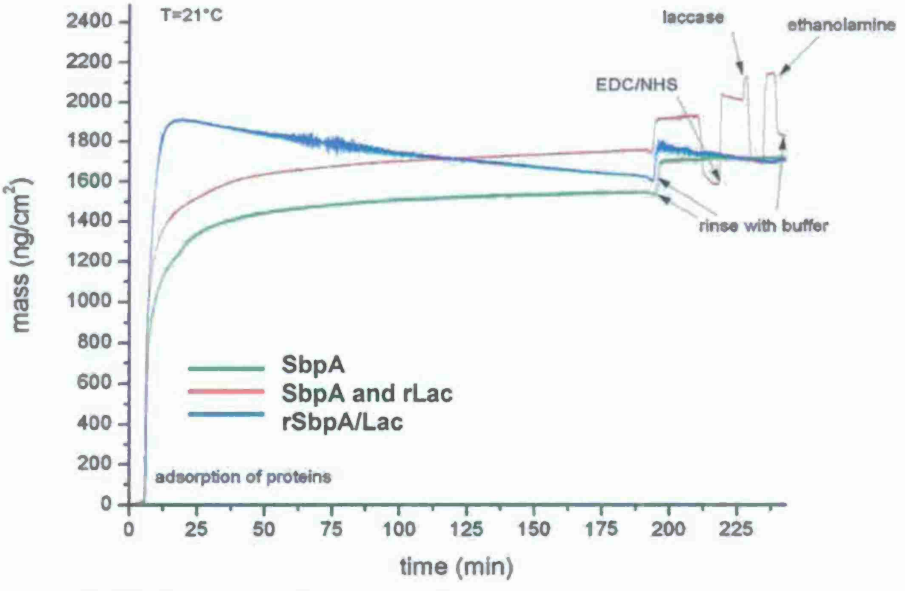


Fig. 16. QCM-D results of adsorbed S-layer protein SbpA from *Ly. sphaericus* CCM 2177 and rSbpA/Lac on a gold sensor and of rLac covalently bound to SbpA.

Table. 4. Maximum laccase activity obtained for immobilized enzymes on the gold sensor (QCM-D) crystal

Substrate	T(°C)	pH	**rLac on S-layer lattice** (units*/mg)	**rSbpA/Lac** (units*/mg)
DMP	45	7.5	51.02 +/- 13.65	6.25 +/- 2.62

Analysis by spectrophotometry.
* 1 U corresponds to the amount of laccase that oxidizes 1 µmol substrate per minute under standard assay conditions. Taking different molecular weights into account; value represents U/mg calculated to the amount of enzymatic groups.

Spectrophotometric measurements: rSbpA/Lac recrystallized on SiO_2 beads

SiO_2-nanoparticles (3 µm, Sigma) were incubated with a solution containing rSbpA/Lac monomers (in crystallisation buffer: 0.5 mM Tris-HCl buffer, 10 mM $CaCl_2$ at pH 9.0) over night at 4 °C. After two washing steps, the beads were resuspended in A. purif. The reaction mixture (100 mM MOPS buffer, supplemented with 100 mM NaCl and the beads coated with rSbpA/Lac) was pre-incubated at 45°C. The reaction was started by adding DMP to a final concentration of 1 mM (*12*). The concentration of the oxidized substrates was calculated using a photometer adjusted to the respective wavelength.

Table 5. Maximum laccase activity obtained for the immobilized fusion protein on SiO_2 beads.

Substrate	T(°C)	pH	**rSbpA/Lac** (units*/mg)
DMP	45	7.5	6.95 +/- 0.33

Stability test

rSbpA/Lac recrystallized on SiO_2 beads showed quite a low activity. Nevertheless, the storage stability was investigated over three weeks. About 80 % of the activity was retained after two weeks and about 50 % of the original activity after three weeks.

Electrochemical measurements: proteins recrystallized on a gold crystal (QCM-D) - amperometric detection

QCM-D was utilized to monitor the adsorption behavior of the fusion protein. By connection with an electrochemical cell it provides the determination of the enzymatic activity via amperometric detection at the reduction potential of quinone. The electrodes were the same as for the defect area determination. In the experiment, two sensors were coated with SbpA, one

was used as blank and the other was functionalized with laccase via reactive ester coupling. On the third sensor, the fusion protein SbpA/Lac was recrystallized. The recrystallization condition for all sensors was 0.5 mM Tris-HCl buffer, 10 mM $CaCl_2$ at pH 9.0. After 3 hours, the cells were flushed with potassium phosphate buffer pH 7.6 and the cell temperature was adjusted to 45°C. For the amperometric reduction of oxidized hydroquinone at -0.2 V, an electrochemical cell (QEM 401, Q-sense) was connected through each cell, then flushed with 1mM hydroquinone solution in the same buffer and incubated for 5 minutes. After incubation, the cells were flushed under a distinct flow and the current was measured against time (*16*). Owing to the fact that QCM-D provides the adsorbed mass, the specific activity can be determined (Tab. 6).

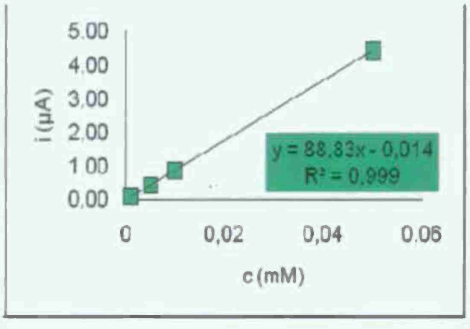


Fig. 17. Calibration graph of the reduction of quinone at 45°C under flow conditions.

The amount of converted substrate was determined by a calibration of the EC cell with respect to the oxidized substrate. A calibration graph was performed as described by (*16*). Quinone was detected at -0.2 V at various concentrations and the current values were plotted against the concentration (Fig. 17).

Table 6. Calculated specific activity from QCM-D data and amperometric detection.

surface	adsorbed mass (mg)	i (µA)	n (mM)	Δc (mM)	substrate (µmol)	units*/mg
rSbpA/Lac	$9{,}71 \times 10^{-4}$	35.6	0.395	0.202	0.0403	6.93
rLac on S-layer lattice	3.07×10^{-5}	22.5	0.25	0.562	0.0112	61.11
SbpA (blank)	2.07×10^{-3}	17.4	0.194	-	-	-

* 1 U corresponds to the amount of laccase that oxidizes 1 µmol substrate per minute under standard assay conditions. Taking different molecular weights into account; value represents U/mg calculated to the amount of enzymatic groups.

Conclusion of rSbpA/Lac results

The aim of this study was to obtain an electrode completely covered with recrystallized rSbpA/Lac fusion protein. As alternative strategies, the construction of an rSbpA/Lac fusion protein with a cysteine residue introduced at the very N-terminal end of the protein sequence, as well as the sole laccase chemically linked to the S-layer lattice was considered. After cloning, expression, isolation and purification of the proteins the enzymatic activity of the enzyme-moiety in the fusion proteins was checked. Interestingly, the S-layer moiety confers a much higher solubility on the laccase as for the sole enzyme without S-layer protein. Spectrophotometric measurements revealed the highest enzymatic activity of the fusion protein with Dimethoxyphenol as substrate at 45°C and pH 7.5. Electrochemical studies revealed the best results concerning activity with Hydrochinone as the substrate at 40°C and pH 7.6. These two activity determinations applied to the enzymes in solution could not be compared with each other because of the incalculable mass of enzyme at the electrode interface in the case of electrochemical measurements. By determining the defect areas of QCM-D gold sensors covered either by rSbpA/Lac or native SbpA, complete coverage could be observed for the fusion protein, although no square lattice structure was detected by atomic force microscopy. Therefore, silicium oxide-beads and gold sensors were coated with SbpA, rSbpA/Lac and/or rLac (covalently linked to the SbpA-layer via amine coupling) in order to calculate the activity of immobilized enzyme compared to enzymes in solution. rSbpA/Lac showed quite a low activity when immobilized on solid supports and revealed the same values in spectrophotometric and electrochemical measurements. The highest activity could be achieved with rLac covalently linked to the SbpA monolayer, which was about double compared to rSbpA/Lac in solution. Again, the spectrophotometric and the electrochemical measurements showed almost the same results. To sum up, the laccase from *B. halodurans* C-125 is an interesting biocatalyst in applications for which classical laccases are unsuited. Concerning S-layers, the preferred use should be the chemical linkage of the enzyme to the S-layer lattice.

Dissemination of the rSbpA/Lac results: **Manuscript in preparation**

S-layer-based biocatalysts carrying multimeric extremozymes

c) rSbpA/XylA fusion protein

In most industrial applications, enzymes require to be immobilized for reuse and facilitated reactor design. This has motivated many researchers to try to associate other improvements of the enzyme properties to this immobilization step, like increased stability, activity or selectivity, or decreased inhibitions. Among these, the prevention of subunit dissociation of multimeric enzymes is one of the most interesting goals (*17*). An exceptional promising new approach can be seen in the production of biocatalysts comprising an S-layer lattice that provides functional multimeric enzymes on its surface thereby guaranteeing the maximal effectiveness and stability of these enzymes. For this proof of concept, the monomer of the tetrameric xylose isomerase XylA from the thermophilic *Thermoanaerobacterium strain* JW/SL-YS 489 was genetically linked to a monomer of the S-layer protein SbpA of *Ly. sphaericus* CCM 2177 via a flexible linker (Fig. 18.). Recrystallization of the subunits of the S-layer fusion protein should lead to the formation of the active enzyme exposed on the surface of the S-layer lattice on solid supports (Fig. 19).

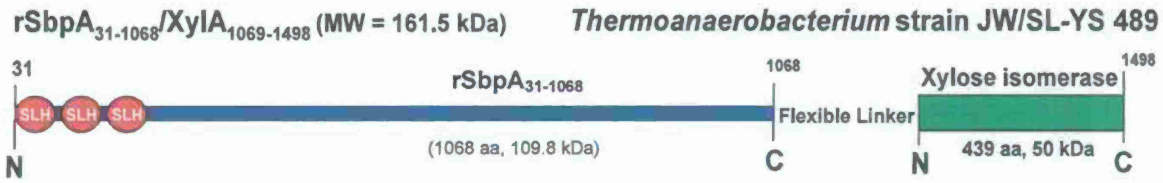


Fig. 18. Schematic representation of the S-layer/extremozyme fusion protein comprising the truncated S-layer protein SbpA, a 20 amino acids long linker, and the xylose isomerase (XylA)

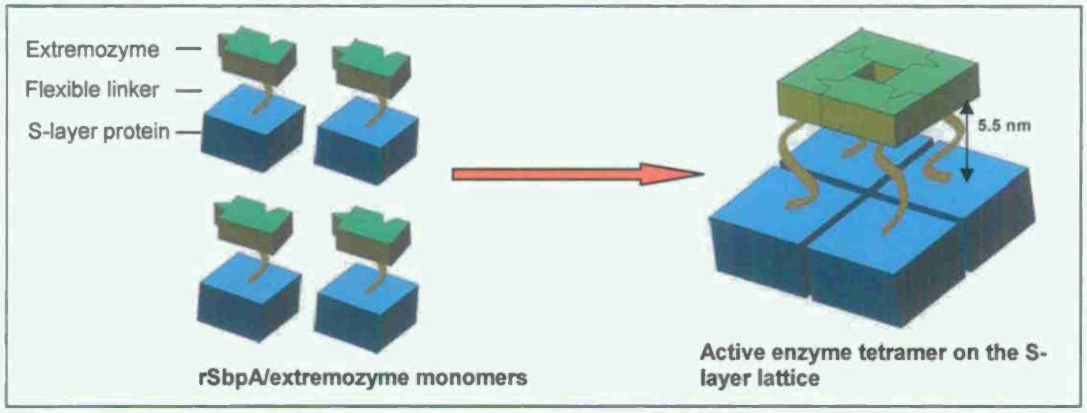


Fig. 19. Schematic drawing of the construction of S-layer-based biocatalysts carrying multimeric extremozymes. The active enzyme is formed by self-assembly of four monomeric subunits of the S-layer/extremozyme fusion protein.

Cloning, expression, isolation and purification of the SbpA/XylA fusion protein and the sole enzyme

The S-layer/extremozyme fusion protein rSbpA/XylA carrying the S-layer protein SbpA and the tetrameric xylose isomerase XylA from the thermophilic organism *T. strain* JW/SL-YS 489 (Liu et al., 1996) and the sole enzyme were cloned and heterologously expressed in *E. coli* (Fig. 18). For the construction of expression vectors, the first intermediate construct was made by using the primer pair *SbpA37/BamHI-SbpA*$_{1068}$*rev* (Tab. 7) to generate a SbpA-PCR fragment encoding amino acids 31-1068 of the SbpA sequence and cloning it into the *Nco*I and *BamH*I sites of plasmid pET-28a(+). The xylose isomerase nucleotide sequence was amplified with the primer pair *XylA-for/NotI-XylA-rev* (Tab. 7) using the chromosomal DNA of *T. strain* JW/SL-YS 489 (purchased from the German Collection of Microorganisms and Cell Cultures) as template. This PCR product was the template for adding the sequence encoding the 20 amino acids long linker (5'-GGA TCC GGG GCG AGC AGC GGC GCG GGG AGC GGA GCG GGG AGC AGC GGC GCG GGA AGC GCG-3') via the primer pair *BamHI-Linker*$_{20}$*-XylA-for/NotI-XylA-rev* (Tab. 7). During PCR, the restriction sites *BamH*I and *Not*I were introduced at the 5' and 3' ends, respectively. The nucleotide sequence for cloning the sole xylose isomerase monomer into pET-28a(+) was amplified by using the primer pair *NcoI-XylA-for/NotI-XylA-rev*. The plasmid and the PCR product were linked together by the restriction enzymes *NcoI* and *NotI*. For expression, all recombinant plasmids were established in *E. coli* BL21 Star™ (DE3) (Invitrogen). After induction of gene expression by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG), samples of

cultures from *E. coli* BL21 Star™ (DE3) were taken at distinct points of time and analyzed by SDS-PAGE (Fig. 20). The proteins could be isolated from the insoluble cytoplasmic fraction and purified by gel-permeation chromatography (GPC) (Fig. 21).

Table 7. Oligonucleotide primers used for amplification in PCR; underlined: overhang, **bold**: restriction sites, *italicized*: start and stop codon

Primer	[1,2] *Primer Sequence 5'to 3'*
XylA-for	*ATG*AATAAATATTTTGAGAATGTATC
NcoI-XylA-for	CGGAATT**CC*ATGG***CGAATAAATATTTTGAGAATGTATC
BamHI-Linker$_{20}$-XylA-for	CGC**GGATCC**GGGGCGAGCAGCGGCGCGGGGAGCGGAGCGGGGAGCAG CGGCGCGGGAAGCGCG*ATG*AATAAATATTTTGAGAATGTATC
NotI-XylA-rev	GGCC**GCGGCCGC***TCA*TTCTGCAAACAAATACTG
*SbpA*37	Ilk *et. al.* 2002
BamHI-SbpA$_{31-1065}$-rev	CGC**GGATCC**TTCTGAATATGCAGTAGTTGCTGC

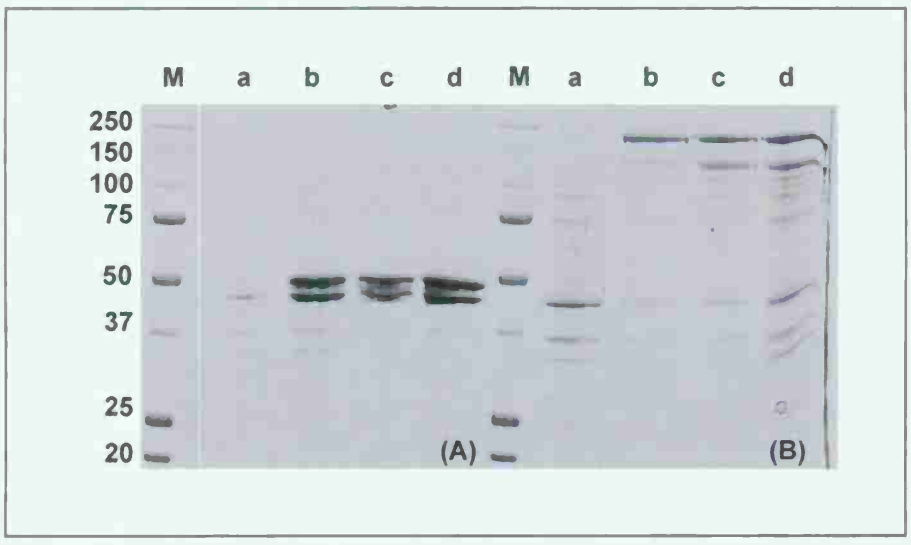


Fig. 20. SDS-PAGE analysis and Coomassie staining of SDS extracts from *E. coli* BL21 Star™ (DE3) expression cultures containing (A) the pET28a/*sbpA/xylA* gene construct before (lane a), and 1, 2 and 3 h after induction of *sbpA/xyla* expression (lanes b, c and d). (B) SDS-PAGE pattern of a whole-cell extract of *E. coli* BL21 Star™ (DE3) harbouring the pET28a/*xylA* gene construct before (lane a), and 1, 2 and 3 h after induction *xylA* expression (lanes b, c and d). Selected molecular masses are given in kDa.

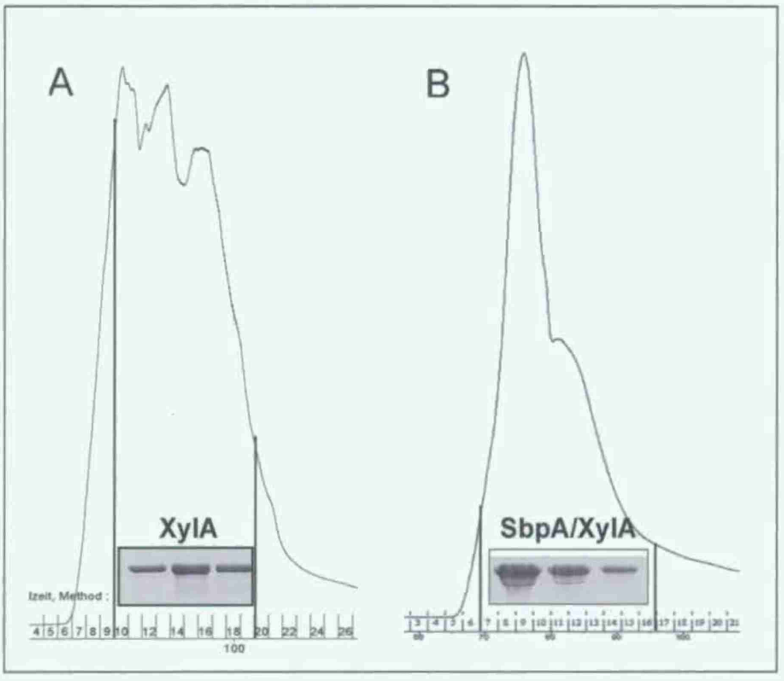


Fig. 21. Purification of rSbpA/Lac by gel permeation chromatography. The protein bands demonstrate the purified proteins in the respective pooled fractions.

Determination of the enzymatic activity of rXylA and rSbpA/XylA

The Xylose isomerase is also referred to as glucose isomerase (D-glucose ketoisomerase) because of its ability to cause the reversible isomerization of D-glucose to D-fructose. Typically, the pH optima of commercially available glucose isomerases range from 7.5 to 9.0. The xylose isomerase from *Thermoanaerobacterium* strain JW/SL-YS 489 has a maximal activity at pH 6.4 (60°C) or pH 6.8 (80°C) in a 30-min assay (*18*).

Enzyme preparation and assay

The lyophilized proteins (rXylA and rSbpA/XylA) were dissolved in 7 M GHCl in 50 mM Tris/HCl buffer (pH 7.2) and dialyzed against A. purif. containing 1 mM cobalt chloride.

The reaction mixture contained 8 mM Glucose, 10 mM $MgSO_4$, 1mM $CoCl_2$ in 0.1 M MOPS buffer adjusted to the respective pH and temperature. Xylose isomerase activity was measured by incubating 900 µl reaction mixture and 100 µl of the enzyme solution. After incubation at 60 °C or 80°C for 30 min, the amount of fructose formed was estimated by the cysteine carbazole sulfuric acid method (*19*). One unit of xylose isomerase activity is defined as the

amount of enzyme required to produce 1 µmol of fructose under the assay conditions described above (Tab. 8).

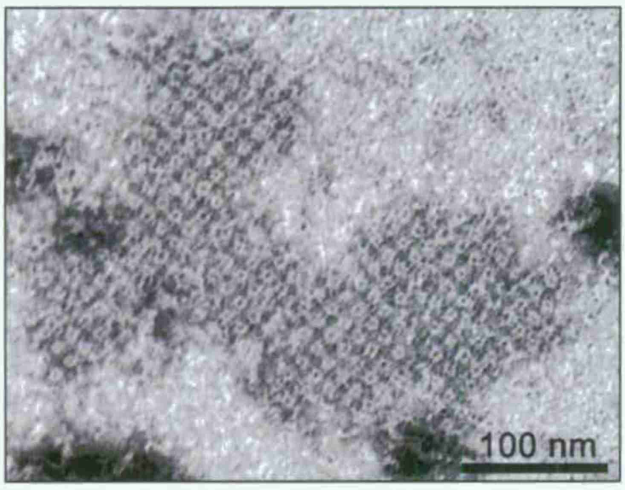
Table 8. Maximal enzymatic activity of rXylA and rSbpA/XylA

T(°C)	pH	rXylA (units*/g of Enzyme)	rSbpA/XylA (units*/g of Enzyme)
60	6.5	4.45 +/- 0.6	4.85 +/- 1.2
80	6.5	35.5 +/- 0.7	35 +/- 4.2
80	6.7	34 +/- 7	27 +/- 7

*Taking different molecular weights into account; value represents U/g calculated to the amount of enzymatic groups

Investigation of the self-assembly and recrystallization properties of rSbpA/XylA

Investigation by transmission electron microscopy of negatively stained preparations revealed that rSbpA/XylA had the capability to self-assemble in suspension into monomolecular arrays (Fig. 22) and in double layered rSbpA/XylA self-assembly product (Fig. 23)



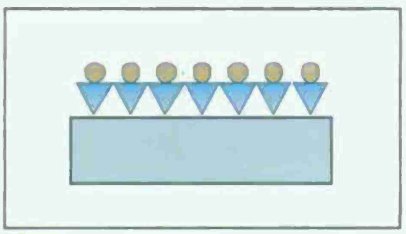
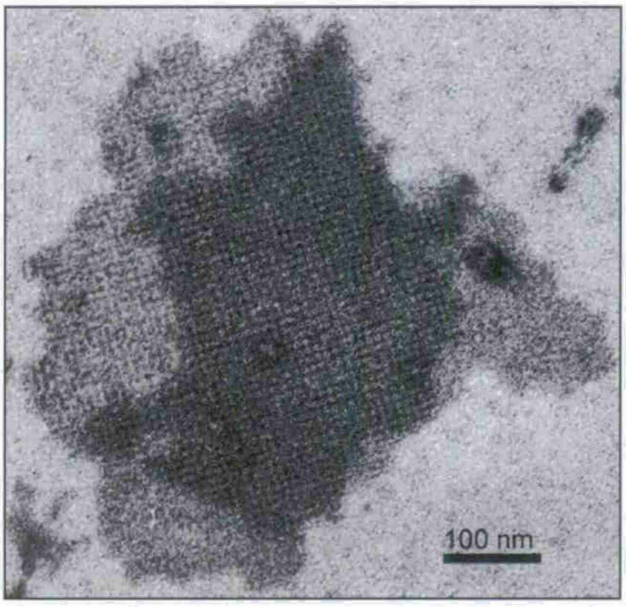


Fig. 22. TEM image of a negatively stained preparation of rSbpA/XylA fusion protein recrystallized as a crystalline monolayer copper grid



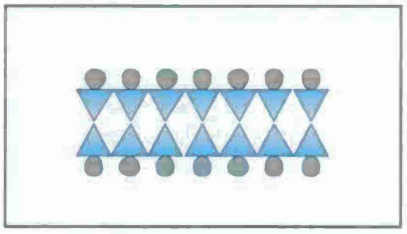


Fig. 23. TEM image of a negatively stained preparation of a double-layered rSbpA/XylA self-assembly product formed in solution

Conclusion of rSbpA/Xyl results

In this part of the project, for the first time, self-assembling biocatalysts consisting of S-layer lattices exhibiting surface exposed active multimeric enzymes of extremophiles, were produced (**Manuscript in preparation**). In the fusion protein, the multimeric enzymatic portion exhibited the same biochemical properties as the sole enzyme concerning pH and temperature optimum. The highest specific xylose isomerase activity of rSbpA/XylA was measured at pH 6.5 and 80°C. The recrystallization of the subunits of the S-layer fusion protein lead to the formation of the active enzyme exposed on the surface of the S-layer lattice.

d) rSbpA/Cam fusion protein

At the 2312DX review in Key West in January 2008, we discussed our planned experiments on multimeric enzymes with Prof. Greg Ferry and he suggested the following cooperation between the Department of Nanobiotechnology, Vienna, Austria and the Department of Biochemistry & Molecular Biology, Pennsylvania State University:

Production of biocatalysts based on fusion proteins between S-layer proteins and multimeric carbonic anhydrases from archaea

The carbonic anhydrase (CA) is a Zn^{2+}-containing enzyme catalyzing the reversible hydration of CO_2 ($CO_2 + H_2O \longleftrightarrow HCO_3^- + H^+$). CA is widely distributed in nature (three distinct classes: CA of mammals = α class, CA from chloroplasts = β class, and CA of *Methanosarcina thermophila* = γ class) and participates on various functions, which include facilitated diffusion of CO_2, interconversion of CO_2 and HCO_3^- during photosynthesis, pH homeostasis, and ion transport (*20*). These metallo-enzymes are among the fastest enzymes described so far having turnover numbers of up to 10^6/s (*21*). In the methanogenic archaeon *Methanosarcina thermophila*, a metabolic switch from methanol to acetate as the energy source results in the expression of a carbonic anhydrase (Cam). The active form of the enzyme is a trimer with three zinc-containing active sites, each located at the interface between two monomers. Cam has an estimated native molecular mass of 84 kDa. N-terminal analysis of the first 26 residues detected only one N-terminal sequence that suggests that the enzyme is composed of identical subunits (*22*). The enzyme is stable to heating at 55°C for 15 min but was inactivated at higher temperatures.

Overexpression, isolation and purification

The S-layer/extremozyme fusion protein carrying the carbonic anhydrase of the archaeon *Methanosarcina thermophila* was cloned and heterologously expressed in *E. coli* (Fig. 24). The fusion protein was isolated from the host cells, purified by gel permeation chromathography (Fig. 25), with an overall yield of 100 mg of purified fusion protein per L expression medium and its recrystallization properties in suspension as well as on solid supports were investigated by TEM and AFM. The fusion protein, catalyzing the hydration of CO_2, showed excellent recrystallization properties in suspension (Fig. 26) and on solid supports (Fig. 27, 28 and 29) as well as good enzymatic activivity indicating that the active Cam-multimer is formed on the crystalline SbpA-lattice (data summarized in Table 9).

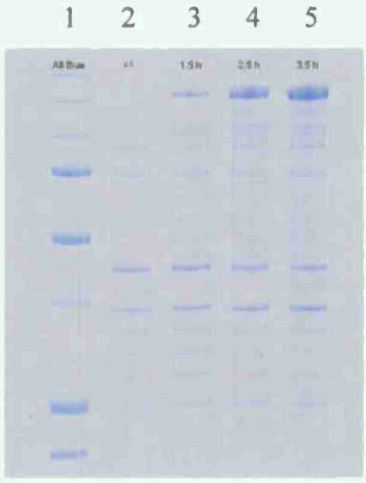


Fig. 24. SDS-PAGE of the overexpression of the rSbpA/Cam fusion protein. Coomassie staining. Lane 1, Benchmark ladder; lane 2, *E. coli* BL21star(DE3) expression culture harboring plasmid pET28a/*sbpA/cam* before induction; lane 3, after 1.5 h , lane 4 after 2.5 h and lane 5 after 3.5 h of induction.

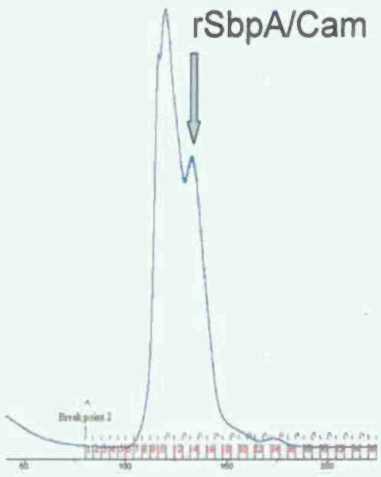
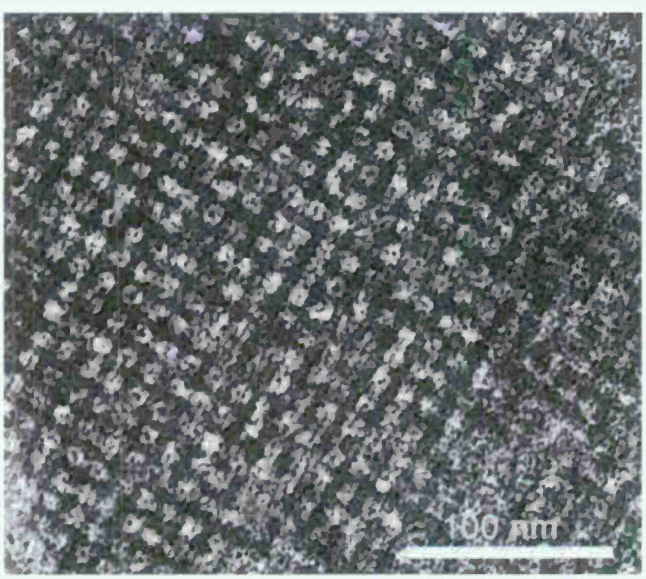


Fig. 25. Purification of rSbpA/Cam by gel permeation chromatography.



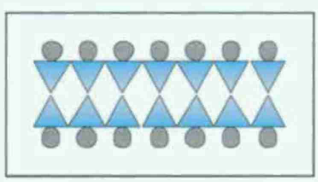
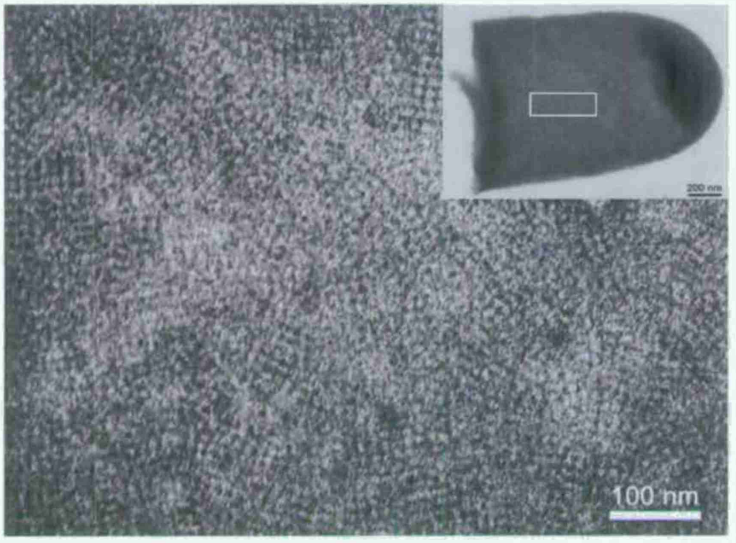


Fig. 26. TEM image of a negatively stained preparation of a double layered rSbpA/Cam self-assembly product formed in solution.



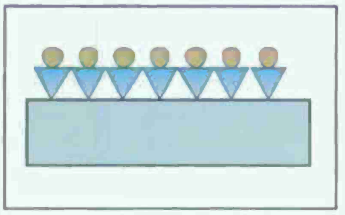
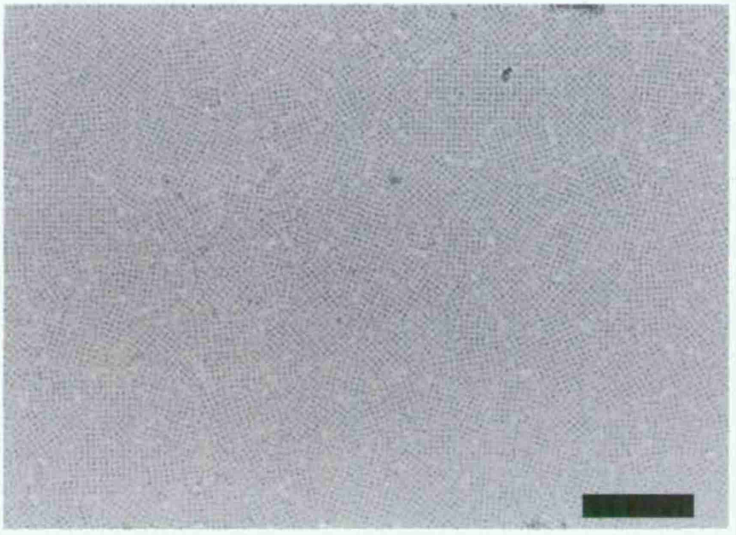


Fig. 27. TEM image of a negatively stained preparation of a crystalline rSbpA/Cam monolayer formed on peptidoglycan-containing sacculi of *Ly. sphaericus* CCM 2177.



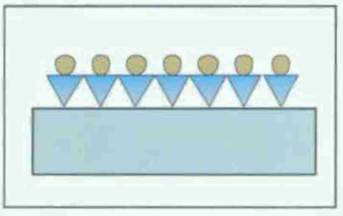
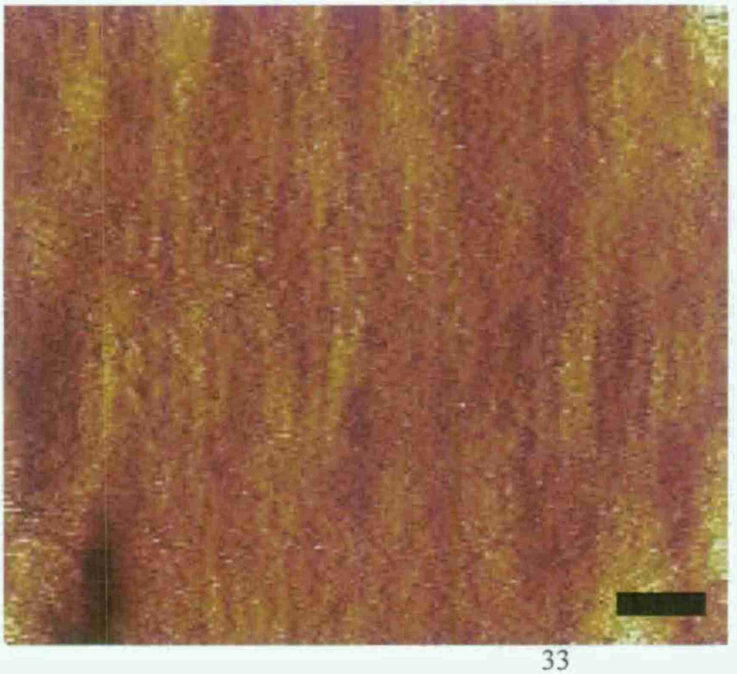


Fig. 28. TEM image of a negatively stained preparation of rSbpA/Cam recrystallized into a monolayer on Formvar-coated copper grids which were stabilized by vaccum deposition of carbon, bar 100 nm.



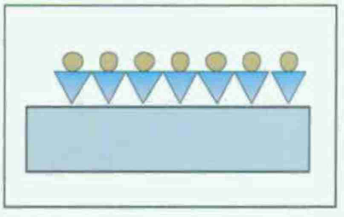


Fig. 29. AFM deflection image of rSbpA/Cam recrystallized on a silicon wafer measured in liquid, oxide-shaped Si3N4 tips, scan rate 4 Hz recorded in contact mode. bar 50 nm.

Investigation of specific enzyme activity rSbpA/Cam

In the Wilbur-Anderson-Test, the determination of the time required for a saturated CO_2 solution to lower the pH of Tris/HCl buffer from pH 8.0 to 6.3 at 0°C is measured.

$$\text{Units/mg} = \frac{2 \times (T_{Blank} - T_{Test})}{T_{Test} \times \text{mg enzyme in reaction mixture}}$$

T_{Blank}: time without enzyme
T_{Test}: time with enzyme

Uncatalyzed reaction: 100 sec (conviently measurable periode)
Catalyzed reaction < 80 sec

By using the Wilbur-Anderson-assay, specific enzyme activity of a) soluble rSbpA/Cam fusion protein, b) rSbpA/Cam self-assembly product as well as c) rSbpA/Cam monolayers on solid supports could be detected. Soluble rSbpA/Cam matched the specific enzyme activity of sole rCam in solution. Immobilized rSbpA/Cam matched the specific enzyme activity of sole rCam in solution. In the recrystallized state, the fusion protein showed higher enzyme activity as the immobilized sole enzyme (Tab. 9.).

Table 9. Enzyme activity of the S-layer fusion protein rSbpA/Cam and sole enzyme rCam.

Specific enzyme activity[1]	**rSbpA/Cam (133 kDa)**[2]	**rCam (28 kDa)**[2]
a) in aqueous solution	water soluble rSbpA/Cam in absence of Ca^{2+} ions **444 U/mg ± 1.9**	**415 U/mg ± 1.7**
b) self-assembly products	Crystalline arrays formed in the presence of Ca^{2+} ions **319 U/mg ± 1.1**	**xxx**
c) Monolayer on solid supports	SbpA/Cam recrystallized **414 U/mg ± 2.0**	rCam attached adhesively **294 U/mg ± 1.3**

1 Measurements were performed in quadruplicate and standard deviations were calculated.
2 Differences in molecular masses of rSbpA/Cam and rCam were taken into account.

rSbpA/Cam stability tests

Freeze dried samples of rSbpA/Cam fully maintained the enzymatic activity, whereas rCam lost 32% of enzymatic activity. Investigation of storage stability revealed that soluble rSbpA/Cam as well as non-cross-linked rSbpA/Cam recrystallized on solid supports (PGS of *Ly. sphaericus* CCM 2177) in MilliQ water at 4°C were stable for at least 6 weeks.

Dissemination of the rSbpA/Cam results: **Manuscript in preparation**

6. BIOCAT Achievements

Self-assembly systems that exploit the molecular scale manufactory precision of biological systems are prime candidates for nanobiotechnology. In the present project, 4 different S-layer/enzyme fusion proteins comprising the S-layer protein SbpA from *Ly. sphaericus* CCM 2177 as well as monomeric (LamA, Lac) or multimeric (XylA, Cam) extremozymes were constructed. Special prurification and refolding protocols for each fusion protein were established. All S-layer/extremozyme fusion proteins showed excellent recrystallization properties. The catalytic domain could be localized on the surface of the S-layer lattice. By exploiting the intrinsic self-assembly property of the S-layer protein moiety, the chimeric proteins provided for spatial control over display of enzyme activity on planar and porous supports. Depending on the fused extremozyme, enzyme assays of recrystallized and cross-linked fusion protein revealed that S-layer/extremozyme fusion proteins immobilized on solid supports showed the same or significant higher enzyme activity in comparison to adhesively or covalently bound native enzyme. Remarkably, after repeated use, S-layer fusion proteins showed improved operational and storage stability in comparison to adhesively bound native enzymes. Additionally, it could be demonstrated that S-layer cushion prevented denaturation of the enzyme upon immobilization. To conclude, in combination with the good self-life and the high resistance towards temperature and diverse chemicals, these novel biocatalysts are regarded a promising approach for site-directed enzyme immobilization.

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Sleytr, U.B. (2008): Development of a Molecular Construction Kit for Nanobiotechnological Applications. PCST-10 Conference: Malmö – Lund – Copenhagen; Session: Visualization in science and public communication, University Malmö. Malmö, Schweden.

Sleytr, U.B. (2008): Nanobiotechnological applications of S-layers. CALIT Visionary Workshop on Biomaterials and Nano-Bio Symposium 2008 – Biomaterials, the first line of tolerability management in the living organism. Leuven, Belgium.

Sleytr, U.B. (2008): S-layer proteins as basic building blocks for nanobiotechnological applications. MIT PPST Polymer Seminars – Fall 2008 Meeting, Center for Biomedical Engineering and Center for Bits & Atoms, Massachusetts Institute of Technology (MIT), Cambridge/Boston, USA.

Sleytr, U. B. (2009): Nanobiotechnology – What is new? 1st International Conference on Biotechnology (ICOB), Center of Excellence in Biotechnology Research, King Fahad Cultural Center, Riyadh, Saudi Arabia.

Sleytr, U. B. (2009) Nanobiotechnology with monomolecular protein lattices. Conference on Membrane Biology Frontiers: Dynamics, Energy, Structure and Technology, Royal Myconian Resort, Mykonos, Greece.

10. FACILITY AND EXPERIMENTAL EQUIPMENT

The research project BIOCAT was performed at the Department of NanoBiotechnology at the University of Natural Resources and Applied Life Sciences Vienna, Austria. The Department of NanoBiotechnology (former Center for Ultrastructural Research, founded in 1980) is headed by Uwe B. Sleytr.

Instruments and equipment used for BIOCAT

Thermocycler (Primus96 advanced, peqlab): PCR, production of chimaeric genes encoding recombinant S-layer/extremozyme fusion proteins

Gene pulser® II (BIORAD): Electroporation of *E. coli* cells

VILBER LOURMAT BIOVISION 3000 (peqlab): Gel documentation system

Fermenter (10 L) BIOSATE® C (B. Braun Biotech International): Expression of recombinant S-layer/extremozyme fusion proteins

Eppendorf 5804R, Eppendorf 5424, Sorval RC6: Centrifugation of samples

Äktaprime plus (GE healthcare): Purification (gel permeation chromatography) of recombinant fusion proteins

SDS-PAGE equipment (BIORAD): Characterization of recombinant S-layer/extremozyme fusion proteins

Tecnai G^2 (FEI) and Philips CM12 (Philips Eindhoven, The Netherlands): Investigation of recrystallization properties of recombinant S-layer/extremozyme fusion proteins by transmission electron microscopy (TEM)

Digital Instruments Nanoscope V (Santa Barbara, CA): Investigation of recrystallization properties of recombinant S-layer/extremozyme fusion proteins on solid supports by atomic force microscopy (AFM)

Biacore®2000 (GE Healthcare): Surface plasmon resonance (SPR) spectroscopy of recombinant S-layer/extremozyme fusion proteins

Quartz crystal microbalance with dissipation monitoring (QCM-D) (Q-Sense AB, Gothenburg, Sweden): Recrystallization of recombinant S-layer/extremozyme fusions proteins on gold sensors and performance of enzyme assays

The equipment was funded by the Austrian government, the Austrian Science Fund, the US Air Force Office of Scientific Research, and the European Commission.

Interactions with other Groups and Organizations

1. CatchMabs BV, Wageningen, NL: provided gene sequence encoding the enzyme LamA from *Pyrococcus furiosus*
2. James G. Ferry, Department of Biochemistry & Molecular Biology, Pennsylvania State University: provided gene sequence encoding the enzyme Cam from *Methanosarcina thermophila*
3. Dietmar Haltrich, Department of Food Sciences and Technology, BOKU, Vienna: provided gene sequence encoding the enzyme Lac from *Bacillus halodurans*

Special test equipment

No special test equipment was required.

High performance computing availability

No high performance computing availability was required.

Human subjects

Research did not involve human subjects.

Animal use

Research did not involve animals.

11. SCIENTISTS WHICH CONTRIBUTED TO THE PROJECT

Prof. Dr. Uwe B. Sleytr, PI

Dipl. Biol. Dr. Nicola Ilk (Senior PostDoc), Co-PI

Mag. Dr. Judith Ferner Ortner Bleckmann (PostDoc)

Mag. Dr. Helga Tschiggerl (PhD)

Manfred Tesraz (Technician)

Mag. Angelika Schrems (PhD)

Prof. Dr. Dietmar Pum